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# Inhibition of Cholesterol Oxidation Products (COPs) Formation in Emulsified Porcine Patties by Phenolic-Rich Avocado (*Persea americana* Mill.) Extracts

Javier-Germán Rodríguez-Carpena,[†] David Morcuende,[‡] María Jesus Petrón,[§] and Mario Estévez*[,‡]

[†]Faculty of Veterinary, Autonomous University of Nayarit, Tepic 63190, Mexico

[‡]Food Technology, Animal Production and Food Science, University of Extremadura, Cáceres 10003, Spain

[§]Laboratory of Food Technology, Escuela de Ingenierías Agrarias, University of Extremadura, Badajoz 06007, Spain

**ABSTRACT:** The effect of phenolic-rich extracts from avocado peel on the formation of cholesterol oxidation products (COPs) in porcine patties subjected to cooking and chill storage was studied. Eight COPs (7$\alpha$-hydroxycholesterol, 7$\beta$-hydroxycholesterol, 7-ketocholesterol, 20$\alpha$-hydroxycholesterol, 25-hydroxycholesterol, cholestanetriol, 5,6$\beta$-epoxycholesterol, and 5,6$\alpha$-epoxycholesterol) were identified and quantified by GC-MS. The addition of avocado extracts (~600 GAE/kg patty) to patties significantly inhibited the formation of COPs during cooking. Cooked control (C) patties contained a larger variety and greater amounts of COPs than the avocado-treated (T) counterparts. COPs sharply increased in cooked patties during the subsequent chilled storage. This increase was significantly higher in C patties than in the T patties. Interestingly, the amount of COPs in cooked and chilled T patties was similar to those found in cooked C patties. The mechanisms implicated in cholesterol oxidation in a processed meat product, the protective effect of avocado phenolics, and the potential implication of lipid and protein oxidation are thoroughly described in the present paper.

**KEYWORDS:** *cholesterol oxidation products, avocado, phenolic compounds, lipid oxidation, protein oxidation*

## INTRODUCTION

Cholesterol is a natural component of the lipid bilayer cell membrane in animal tissues. It consists of four fused rings and various functional groups that are susceptible to oxidation, leading to the formation of a variety of cholesterol oxidation products (COPs).[1] These compounds, also called oxycholesterols or oxysterols, contain an additional hydroxyl, epoxide, or keto group at the cholest-5-en structure or a hydroxyl group at the side chain of the molecule.[2] In recent years, cholesterol oxidation in foods has gained considerable attention owing to the adverse effects of COPs on health. These compounds have been well documented for being potentially cytotoxic, mutagenic, carcinogenic, and accelerators of fatty streak lesion formation and promotion of atherosclerosis.[3] In meats, the manufacture and/or processing conditions (heating, long-term storage, or packing conditions) promote the oxidation of unsaturated fatty acids through the development of free radicals and peroxides, which accelerate the formation of COPs.[4] Cholesterol oxidizes by a free radical mechanism involving the removal of a labile hydrogen from the molecule by peroxy or oxyradicals of unsaturated fatty acids. Oxidation of cholesterol can occur at $C_7$, $C_{20}$, and $C_{25}$ (Figure 1). The pathway of cholesterol oxidation at the $C_7$ atom is initiated by hydrogen abstraction, followed by the formation of 7$\alpha$- and 7$\beta$-hydroperoxycholesterol (7-OOH) through free radical chain reaction, 7$\alpha$- and 7$\beta$-hydrocholesterol (7-OH) as a result of reduction of these peroxides, and 7-ketocholesterol (7-keto) from 7-hydroperoxycholesterol through dehydration. Both isomeric 7$\alpha$-OH and 7$\beta$-OH can undergo dehydrogenation to 7-keto.[1,5] 7-Ketocholesterol is probably the most common COP in food systems and is usually employed as a marker of cholesterol oxidation. Special attention has also been given to the 5,6-epoxide isomers as they are particularly harmful and are precursors of a triol form that is reported to be the most toxic COP.[1]

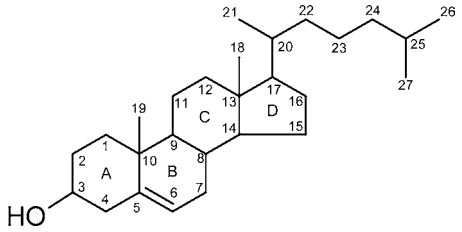


**Figure 1.** Molecular structure of cholesterol.

Animal-source foods, such as muscle foods, are particularly susceptible to oxidation owing to their relatively large amounts of susceptible targets (unsaturated fatty acids and cholesterol) and oxidation promoters (iron, myoglobin). Various COPs accumulate in meat as a result of the application of conventional technologies such as cooking.[13] Modern strategies to control cholesterol oxidation in animal-source foods involve the addition of either synthetic or natural antioxidants directly to the food product and/or the enhancement of the oxidative stability of animal tissues through dietary means.[6] Because cholesterol oxidation proceeds via a free radical mechanism similar to unsaturated fatty acid oxidation, the antioxidants used

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to inhibit lipid oxidation in foods may also be able to prevent or retard cholesterol oxidation. Although the connection between lipid and cholesterol oxidation has been documented, the role of the oxidation of other food components, such as proteins, is mostly unknown. Many studies have addressed the efficacy of synthetic or natural antioxidants as inhibitors of lipid and protein oxidation in processed and cooked meats.[7−9] Only a few, however, have focused on the antioxidant effect and mode of action of plant polyphenols against COPs formation in food systems.[10−12] Plant phenolics are compounds of increasing interest among consumers and researchers owing to their efficacy as natural antioxidants in vitro as their potential role as compounds with beneficial biological effects.[2] Avocado has been recently found to be a rich source of phenolic compounds with intense antioxidant activity against lipid and protein oxidation.[14,15] However, the potential efficacy of avocado phenolics against cholesterol oxidation in a complex food system has been ignored.

The aim of the present paper was to evaluate the effect of avocado phenolic-rich extracts on the formation of COPs in porcine patties subjected to cooking and chilling procedures. The likely mechanisms by which avocado phenolics may inhibit cholesterol oxidation are thoroughly described, and the potential implication of muscle lipids and proteins is also discussed.

## MATERIALS AND METHODS

**Materials.** All chemicals were supplied from Panreac Química, S.A. (Barcelona, Spain), Merck (Darmstadt, Germany), and Sigma-Aldrich Chemicals (Steinheim, Germany). The gases used in GC-MS (helium) and evaporation of solvents (nitrogen) were supplied by Abelló Linde S.A. (Barcelona, Spain). Porcine longissimus dorsi muscle and porcine back fat were purchased from a butchery in Cáceres (Spain), whereas 'Hass' avocado fruits were bought from a local supermarket in Madrid (Spain).

**Methods.** *Phenolic-Rich Avocado Extracts.* Peels from 'Hass' avocado were manually separated from fullly ripened fruits and frozen (−80 °C) until manufacture of extracts. These extracts were obtained from 10 g of peel treated twice by 30 mL of acetone/water (70:30 v/v). Samples and solvents were homogenized using an Omni-mixer homogenizer (model 5100). The homogenates were centrifuged at 700*g* for 3 min at 4 °C. The supernatants were collected with filter paper, and the residue was re-extracted once more following the procedure previously described. The two supernatants were combined and evaporated using a rotary evaporator at around 40 °C, dispensed in 50 mL volumetric flasks, and brought to volume with distilled water. Then water solutions from avocado peels were stored under refrigeration until used (<24 h). According to preliminary sensory tests and the existing literature, peel extracts were not found to display sensory or toxicological concerns advising against their use in food systems. The resulting avocado extract had a considerably high total phenolic content (6082 ± 863 mg GAE/100 g dry matter) with catechins (237.8 ± 4.2 mg/100 g dry matter), hydroxycinnamic acids (282.7 ± 6.9 mg/100 g dry matter), flavonols (1.7 ± 2.5 mg/100 g dry matter), and procyanidins (4592.0 ± 129.4 mg/100 g dry matter) being the major components.[15] According to a previous study,[15] this extract exhibits an intense scavenging activity against both CUPRAC and DPPH radicals (275.36 ± 59.09 and 130.26 ± 36.80 mmol Trolox/g fresh matter, respectively).

*Manufacture of Porcine Patties.* Ingredients per kilogram of porcine patty (control patty, C) were as follows: 700 g of porcine longissimus dorsi muscle, 180 g of distilled water, 100 g of porcine back fat, and 20 g of sodium chloride. Patties were elaborated following the process described by Ganhão et al.[9] Patties treated with avocado extracts (T) were produced by replacing in the aforementioned recipe 50 g of the distilled water by 50 g of the water avocado extract. Eighteen patties for each C and T group were produced and divided into three different sets of patties depending on the processing applied to them: raw (R), cooked (CO), and cooked and chilled (CC) patties ($n$ = 6 per avocado and processing treatment). R patties were vacuum packaged and subsequently frozen (−80 °C) the day of manufacture until the analytical experiments (<4 weeks). C patties were cooked at 170 °C for 18 min in a forced-air oven and allowed to cool at room temperature. CC patties were, upon cooking, dispensed in polypropylene trays wrapped with PVC film (oxygen permeability ∼ 17 $cm^3/m^2$ day atm; moisture permeability < 5 $g/m^2$ day; Tecnodur S.L., Valencia, Spain) and stored for 15 days at 5 °C under white fluorescent light (1620 lx), reproducing retail display conditions. CO and CC samples were also vacuum-packaged and subsequently frozen (−80 °C) until required for analysis (<4 weeks). R (moisture, 71.5%; protein, 17.3%; fat, 10.0%) and CO patties (moisture, 62.5; protein, 22.1%; fat, 14.3%) from all groups and treatments had similar proximate compositions and cooking losses (21.7%).[16,17]

*Cholesterol Quantification in Cooked Burger Patties.* The method proposed by Guardiola et al.[18] was followed for the cold saponification of fat and subsequent isolation of cholesterol from the unsaponifiable extract. An internal standard (2.5 mg) (5$\alpha$-cholestane) was used for quantification purposes. The cholesterol from the samples and the internal standard were silanized at room temperature for 30 min by mixing 25 $\mu$L of the unsaponifiable extract, 25 $\mu$L of the standard solution, and 50 $\mu$L of bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BFSTA) (supplied by Sigma, St. Louis, MO). Cholesterol was identified using 5-cholesten-3$\beta$-ol as cholesterol standard and determined by GC using a Hewlett-Packard HP-5890-II chromatograph, equipped with a flame ionization detector and a fused silica capillary column (12 m × 0.2 mm i.d.) with a film thickness of 0.33 $\mu$m and a stationary phase of methyl silicone. Helium was used as carrier gas at a flow rate of 1.2 mL/min. The oven temperature program was from 210 to 264 °C at 5 °C/min, from 264 to 290 °C at 7 °C/min, and 2 min at 290 °C. The injector and detector temperatures were 280 and 290 °C, respectively. The split ratio was 1:25. The inlet pressure was 14 psi, and the sample volume injected was 2 $\mu$L.

*Isolation of COPs from Porcine Patties.* COPs were extracted from porcine patties according to the procedure described by Petrón et al.[19] First, total fat was extracted from porcine patties with a chloroform/methanol solution (1:2 v/v) following the procedure of Bligh and Dyer.[20] Then, the organic phase was evaporated, first in a rotary evaporator at 35 °C temperature and finally under nitrogen. Ten milligrams of fat and 5 mL of hexane/diethyl ether (95:5) were added to a silica-SPE cartridge (Sep-Pak, Waters, Barcelona, Spain). After removal of the nonpolar lipids and the cholesterol fraction by three successive washes with mixtures of hexane/diethyl ether (10 mL, 95:5; 25 mL, 90:10; 15 mL, 80:20), COPs were extracted with 10 mL of acetone. Then, acetone was removed under vacuum at 25 °C on a rotary evaporator. An additional purification step was performed by cleaning up the sample with hexane/ethyl acetate (9:1) in a $NH_2$-SPE cartridge (Varian, Harbor City, CA). COPs residue was silanized with 50 $\mu$L of Sylon HTP hexamethyldisilazane (HMDS) plus trimethylchlorosilane (TMSC) plus pyridine, 3:1:9 (Supelco, Bellefonte, PA). The mixture was kept at 60 °C for 1 h and then cooled until room temperature. COPs silanized were evaporated to dryness under nitrogen and were then redissolved with 50 $\mu$L of chloroform.

*Identification and Quantitative Determination of COPs.* COPs were analyzed by gas chromatography−mass spectrometry (GC-MS) on a Hewlett-Packard HP-6890II chromatograph, equipped with a Hewlett-Packard 5973A mass selective detector and a capillary column SGL-1 (Sugelabor SA, Madrid, Spain) (30 m × 0.25 mm i.d.) with a film thickness of 0.33 $\mu$m and a stationary phase of methyl silicone. Helium was used as carrier gas at a flow rate of 0.54 mL/min. The oven temperature program was 230 °C for 2 min, from 230 to 290 °C at 5 °C/min, and 10 min at 290 °C. The injector and detector temperatures were 270 and 280 °C, respectively. The inlet pressure was 12 psi, and the sample volume injected was 1 $\mu$L. The injection

**Table 1. Cholesterol Oxides Content in Raw (R), Cooked (CO), and Cooked and Chilled (CC) Patties Manufactured as Control (C) or Using Peel Avocado Extracts (T)[a]**

| | R | | CO | | CC | | *p* value[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | T | C | T | C | T | P | A | P × A |
| 7α-OH | LOD[c] | LOD | 38.6b ± 3.1 | 37.8b ± 5.2 | 281.6a ± 17.3 | 41.1b ± 6.6 | <0.001 | <0.001 | <0.001 |
| 7β-OH | LOD | LOD | 48.7bc ± 2.4 | 26.4c ± 3.2 | 353.4a ± 29.3 | 60.1b ± 5.3 | <0.001 | <0.001 | <0.001 |
| 7-keto | LOD | LOD | 83.4b ± 16.8 | 25.9c ± 10.3 | 584.8a ± 18.8 | 82.8b ± 13.1 | <0.001 | <0.001 | <0.001 |
| 20α-OH | LOD | LOD | LOD | LOD | LOD | LOD | | | |
| 25-OH | LOD | LOD | LOD | LOD | <LOQ[d] | LOD | | | |
| triol | LOD | LOD | LOD | LOD | LOD | LOD | | | |
| 5,6β-EP | LOD | LOD | 29.6b ± 4.9 | LOD | 106.3a ± 25.6 | 33.5b ± 5.4 | <0.001 | | |
| 5,6α-EP | LOD | LOD | 7.2b ± 1.6 | LOD | 25.5a ± 5.6 | 8.0b ± 0.3 | <0.001 | | |

[a]Results are expressed in μg cholesterol oxides/100 g patty as the mean ± standard deviation. Values with different letters (a−c) within a row are significantly different ($p < 0.05$). [b]Effect of processing treatments R, CO, and CC (P), addition of avocado extract C vs T (A), and the corresponding interaction (P × A). [c]Limit of detection. [d]Limit of quantification.

was performed in splitless mode. The MS detector was run in full-scan mode. Cholesterol oxides were identified in the mass range ($m/z$) from 100 to 500. All samples were analyzed in selected ion monitoring (SIM) mode for quantification purposes of the compounds, in which the ions of 7α-hydroxycholesterol ($m/z$ 456.3), 7β-hydroxycholesterol ($m/z$ 456.3), 7-ketocholesterol ($m/z$ 472.3), 20α-hydroxycholesterol ($m/z$ 201.2), 25-hydroxycholesterol ($m/z$ 131.1), cholestanetriol ($m/z$ 403.2), 5,6β-epoxycholesterol ($m/z$ 445), and 5,6α-epoxycholesterol ($m/z$ 366) were selected as the most characteristic of the oxides of cholesterol standards. The amount of each COP was calculated using calibration curves with standard compounds (0.1−10 ng/μL) analyzed separately from the sample under identical conditions. It was used to facilitate the qualitative identification and quantitative determination of the sample components.

*Determination of TBARS Numbers.* Thiobarbituric acid-reactive substances (TBARS) were assessed using the method described by Ganhão et al.[21] with some modifications. Briefly, 5 g of patty was dispensed in cone plastic tubes and homogenized with 15 mL of perchloric acid (3.86%) and 0.5 mL of BHT (4.2% in ethanol). During homogenization, the plastic tubes were immersed in an ice bath to minimize the development of oxidative reactions during extraction of TBARS. The slurry was filtered through filter paper and centrifuged (100*g* for 4 min), and 2 mL aliquots were mixed with 2 mL of thiobarbituric acid (0.02 M) in test tubes. The test tubes were placed in a boiling water bath (100 °C) for 45 min together with the tubes from the standard curve. After cooling, the absorbance was measured at 532 nm. The standard curve (8−50 nmol) was prepared using a 1,1,3,3-tetraethoxypropane (TEP) solution in 3.86% perchloric acid.

*Determination of Hexanal.* Hexanal was used an indicator of lipid oxidation and analyzed from the headspace of R, CO, and CC patties by using solid-phase microextraction (SPME) and GC-MS following the method described by Estévez et al.[22] Hexanal was positively identified by comparing its mass spectra and retention time with those displayed by the standard compound (Sigma-Aldrich). The result from the hexanal analysis was provided in arbitrary area units (AAU).

*Determination of Total Protein Carbonyls.* Total protein carbonyls were quantified in patties according to the DNPH method described by Ganhão et al.[9] Protein concentration was calculated from absorption at 280 nm using BSA as standard. The amount of carbonyls was expressed as nanomoles of carbonyl per milligram of protein using an absorption coefficient of 21.0 $nM^{-1}$ $cm^{-1}$ at 370 nm for protein hydrazones.

*Statistical Analysis.* Six patties per treatment (C and T) and technological process (R, CO, and CC) were prepared in independent processes and used as experimental units. Data from experiments were analyzed by analyses of variance (ANOVA) using a two-way model. When a significant effect ($p < 0.05$) was detected, the comparative analyses between means were conducted using the Tukey test. Statistical analyses were performed by using SPSS (v. 15).

## RESULTS AND DISCUSSION

**COPs in Porcine Patties.** The average amounts of cholesterol in R (74.5 ± 10.2 mg/100 g patty), CO (77.4 ± 11.5 mg/100 g patty), and CC patties (71.7 ± 9.2 mg/100 g patty) are within the common range for this type of meat product. As expected, the addition of the avocado extract did not affect the amount of cholesterol in the patties. Under particular processing and storage conditions, this susceptible molecule is oxidatively degraded to yield various COPs. The concentration of COPs in R, CO, and CC porcine patties is displayed in Table 1. No COPs were detected in R samples, whereas successive increases were found to occur upon cooking and the subsequent chilled storage. Among the total eight COPs investigated, five of them, namely, 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7-keto) 5,6α-epoxycholesterol (5,6α-EP), and 5,6β-epoxycholesterol (5,6β-EP) were detected and quantified in the present samples. In general, only three COPs (7α-OH, 7β-OH, and 7-keto) were consistently present in detectable quantities in all samples subjected to CO and CC processes. The predominance of the 7β-isomer observed in the majority of the cases over the 7α-isomer accounts for radical-mediated cholesterol oxidation, because cholesterol epoxidation is marked by β-stereoselectivity.[23] 7-Keto was, by far, the most abundant COP. This compound occurs in relatively high concentrations in many foods, and it has been proposed as an indicator of cholesterol oxidation.[24−26] Several mechanisms lead to the formation of 7-keto, including direct cholesterol oxidation in the presence of ROS and oxygen and also the free radical-induced dehydrogenation of 7-OHs.[27] However, quantities of 25-OH, 20-αOH, and triol were not detected (trace) in these samples. Probably because the lateral chain of the cholesterol is more stable than the nuclei that contain one Δ5-double bond, the free radical attack could initiate in the nuclei, retarding the formation of 20-αOH and 25-OH, so the amounts found of these compounds are negligible. Iron is known to be the most potent initiator of oxidative reactions in meat systems.[28] The redox cycle between the reduced and oxidized forms of this transition metal ($Fe^{3+}/Fe^{2+}$) imparts important catalytic properties including the formation of numerous ROS such as the hydroxyl radical, which is able to initiate cholesterol oxidation. Besides ROS, lipid-derived peroxides are known to be involved in the formation of particular COPs, namely, the epoxy isomers, 5,6α-EP and 5,6β-EP, both detected in the present study. The chemical mechanisms likely implicated in the formation of COPs in the meat samples from the present


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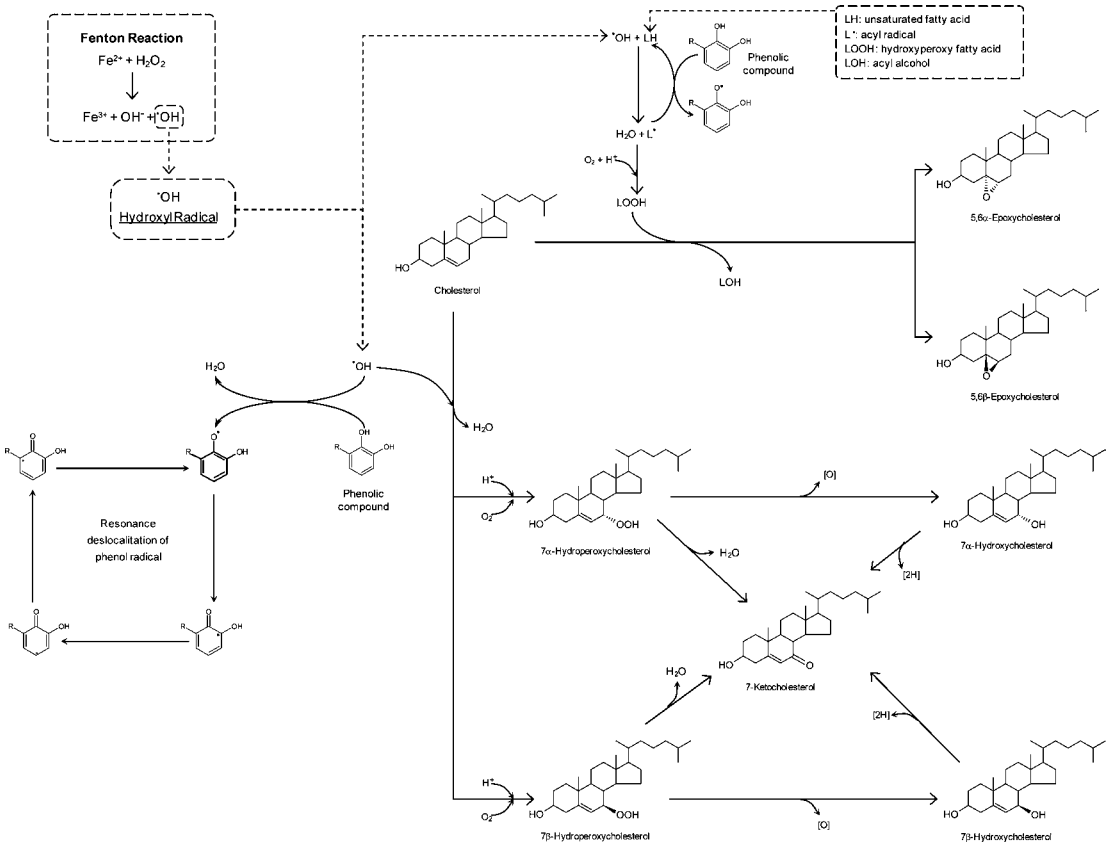


**Figure 2.** Pathways of COPs formation and proposed mechanisms for antioxidant action of avocado phenolics against cholesterol oxidation.

**Table 2. TBARS, Hexanal, and Protein Carbonyls in Raw (R), Cooked (CO), and Cooked and Chilled (CC) Patties Manufactured as Control (C) or Using Peel Avocado Extracts (T)[a]**

| | R | | CO | | CC | | *p* value[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | T | C | T | C | T | P | A | P × A |
| TBARS[c] | 0.24c ± 0.04 | 0.15c ± 0.04 | 0.56b ± 0.03 | 0.29c ± 0.04 | 1.55a ± 0.03 | 0.44b ± 0.04 | <0.001 | <0.001 | <0.001 |
| hexanal[d] | 0.88d ± 0.21 | 0.30d ± 0.17 | 161.63b ± 29.16 | 97.04bc ± 56.66 | 290.51a ± 75.91 | 229.59ab ± 34.25 | <0.001 | 0.736 | 0.428 |
| P-H[e] | 1.59bc ± 0.37 | 1.31c ± 0.34 | 1.87b ± 0.32 | 1.69bc ± 0.26 | 3.42a ± 0.65 | 3.19a ± 0.49 | <0.001 | <0.004 | <0.890 |

[a]Results are expressed as the mean ± standard deviation. Values with different letters (a–c) within a row are significantly different ($p < 0.05$). [b]Effect of processing treatment, R, CO, and CC (P), addition of avocado extract, C vs T (A), and the corresponding interaction (P × A). [c]Expressed as mg MDA/kg patty. [d]Expressed as arbitrary area units (AAU). [e]Protein hydrazones; expressed as nmol hydrazones/mg protein.

study are described in Figure 2. The nature and quantities of the COPs detected in the present study are in line with studies on pork and chicken meat subjected to different cooking methods.[13,23,27] Our results are also consistent with results described for dry-cured ham, which showed higher levels of 7*α*-OH and 7*β*-OH than of other COPs.[19] In general, the patties containing the added avocado extract (T patties) contained smaller concentration of COPs than the C counterparts.

**Effect of Cooking and Chilling on COPs Formation.** Cooking induced the formation of 7*α*-OH, 7*β*-OH, 7-keto, 5,6*β*-EP, and 5,6*α*-EP in porcine patties. Detectable amounts of the two latter were found only in cooked C patties. Right after cooking, T patties had significantly lower amounts of 7-keto than C patties. In general, the amounts of COPs reported in the present study are consistent with those found by Pie et al.[26] in cooked meats. In contrast, Broncano et al.[13] recently found considerably greater levels of COPs in meats cooked following several procedures (grilled, fried, microwaved, and roasted). On the other hand, Janoszka[29] reported lower levels of COPs in pork subjected to more severe cooking conditions. The apparent disparity of values found in the literature suggests that the origin of the raw material, the cooking conditions, and the methodology for the detection of COPs are highly influential on the final quantitative results. It is, however, generally accepted that high temperatures reached during meat cooking accelerate the autoxidation of polyunsaturated fatty acids (PUFA) and, in consequence, the generation of COPs. Because PUFA-containing phospholipids and cholesterol are closely associated as components of the lipid bilayer of the cell membrane, the hydroperoxides derived from PUFA oxidation are believed to play an important role in facilitating cholesterol oxidation.[1] The connection between PUFA oxidation and formation of COPs has been reported in meat products.[13,30] It is particularly remarkable the positive and significant correlations found in the present study between lipid oxidation products (TBARS) (Table 2) and the epoxy isomers, 5,6*α*-EP ($r = 0.65$, $p < 0.05$) and 5,6*β*-EP ($r = 0.62$, $p < 0.05$), as these COPs are usually formed in the presence of peroxides. These results indicate that lipid hydroperoxides are plausibly contributing to the promotion of the formation of particular


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COPs with severe toxic potential. Lipid oxidation has also been recurrently reported to occur concomitantly with the oxidation of another major muscle component: proteins.[31] In fact, a significant correlation was found between TBARS and hexanal and the protein oxidation index (DNPH method) ($r = 0.70$, $p < 0.05$; and $r = 0.60$, $p < 0.05$, respectively). The oxidation of meat proteins is an emerging topic of study,[32] and to our knowledge, the potential connection between protein oxidation and the formation of COPs has never been considered before. The lack of significant correlations between the protein oxidation measurement and COPs indicates that oxidizing proteins may play, if any, a secondary role in the formation of COPs.

As expected, the highest concentrations of COPs were found in cooked and chilled patties (Table 1). The concentrations of COPs in cooked C patties increased between 3- and 7-fold after 15 days of chilled storage. The increase observed in cooked T patties during the same period (from 1- to 3-fold the initial values) was noticeably more moderated. 7-Keto underwent the highest increase, with this compound being the most abundant in cooked and cooked and chilled patties (Figure 3). These

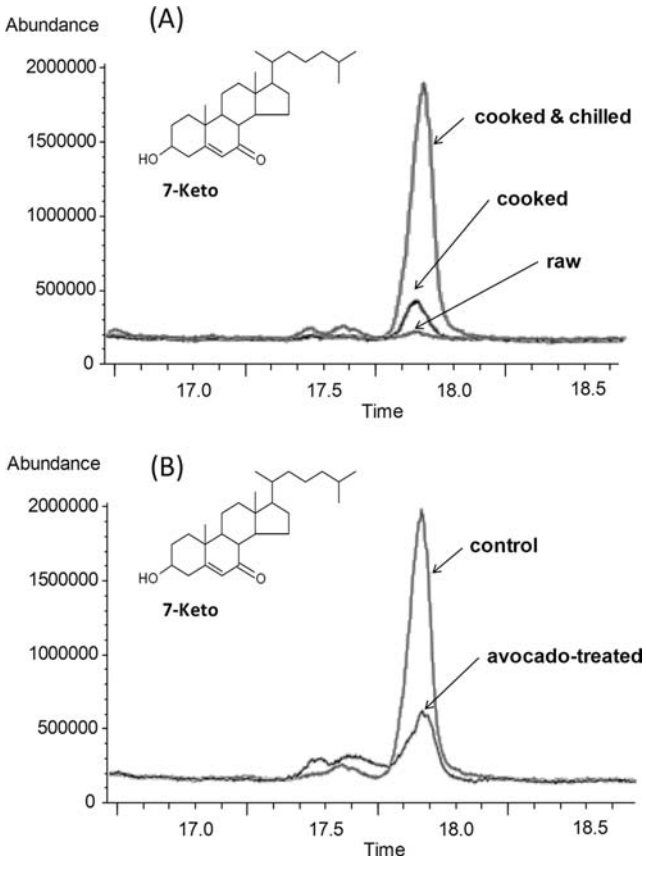


**Figure 3.** Extracted ion chromatograms for 7-ketocholesterol analyzed in porcine patties: (A) influence of the processing technologies in C patties; (B) effect of the phenolic-rich avocado extract in cooked and chilled patties.

results show that the cooking procedure not only promoted the formation of COPs but also promoted the creation of an intense pro-oxidant environment in which cholesterol was readily oxidized during the subsequent chilling storage. The cooking-induced changes leading to such severe pro-oxidant conditions including (i) protein denaturation, which can cause the release of iron from metalloproteins (mainly myoglobin); (ii) disruption of cell membranes, which brings PUFA into contact with pro-oxidants; (iii) transformation of myoglobin into a pro-oxidant specie; and (iv) thermal decomposition of hydroperoxides to pro-oxidant species, such as alkoxyl and hydroxyl radicals.[24,27,33] In addition, the cooked patties from the present study were stored under fluorescent light which could also have promoted the photo-oxidation of cholesterol and hence, the increase of COPs.[11] Similar mechanisms could be ascribed to the results obtained for lipid and protein oxidation measurements (Table 2). These observations are consistent with findings of Pie et al.[26] in cooked and chilled pork. However, our results contrast with the considerations reported by Tai et al.[11] who claimed that low temperatures result in an effective strategy to reduce COPs formation in food systems. According to the present results, cholesterol oxidation in cooked meats is not inhibited by low temperatures but actually highly promoted by the physicochemical changes occurred in patties during the previous cooking. Whereas refrigeration during a limited period of time could delay cholesterol oxidation in certain food systems, the present results advice against using such technology for preserving cooked meats against COPs formation.

**Effect of Avocado Extracts on COPs Formation.** The addition of the avocado extracts to porcine patties significantly inhibited the formation of COPs during cooking and the following chilled storage. In particular, avocado extracts significantly reduced the formation of 7-keto and avoided the formation of the harmful epoxycholesterols during cooking. The major benefit, however, was observed during the subsequent chilled storage. T patties subjected to cooking and chilling had between 3- and 7-fold lower amounts of COPs than the C counterparts (Figure 3). In fact, the final level of COPs in cooked and chilled patties treated with avocado extract was equivalent to that displayed by freshly cooked C patties. The avocado extract also showed an efficient protecting effect toward PUFA in accordance with the TBARS values obtained. The antioxidant effect of the peel avocado extract could be ascribed to its high concentration of polyphenols, namely, catechins, hydroxycinnamic acids, and procyanidins.[15] Avocado phenolics are able to scavenge fatty acyl peroxide radicals and inhibit lipid peroxidation, as demonstrated by Wang et al.[14] and Rodríguez-Carpena et al.[15] The present results confirm the efficacy of these compounds to inhibit the formation of COPs during cooking and chilling of porcine patties, including the highly toxic 5,6-EPs. Avocado phenolics could have protected against the direct oxidation of cholesterol by scavenging ROS or by inhibiting lipid oxidation and, hence, the pro-oxidant effect of lipid-derived hydroperoxides on cholesterol (Figure 2). Consistently, avocado phenolics also significantly reduced TBARS in T patties during cooking and chilling. Particular avocado phenolics (i.e., catechins) could also have prevented cholesterol oxidation by chelating transition metals such as iron and, hence, inhibiting its catalytic actions. This mechanism has been previously ascribed to catechins having antioxidant effects on muscle lipids and proteins.[9,34]

The available information regarding the protective effects of plant phenolics against cholesterol oxidation is considerably limited. Successful results were also obtained by Britt et al.[10] using cherry tissue on beef patties, Janozska[29] using onion and garlic in pork, and Polak et al.[35] using tocopherols and ascorbic acid on chicken liver pâté. The supplementation in the diet with antioxidant compounds such as $\alpha$-tocopheryl acetate seems to

be as well a feasible strategy to inhibit cholesterol oxidation as reported by Grau et al.[27] and Eder et al.[30]

In conclusion, this study demonstrated that peel avocado extract contains active antioxidant compounds that prevent the rapid formation of oxidation products in porcine patties during cooking and chilled storage. The use of phenolic-rich extracts could be an appropriate strategy to elaborate processed meat products with a reduced content of COPs. However, further research must be conducted to provide a better understanding of what compounds from the avocado extract are responsible for the inhibition of cholesterol oxidation.

## AUTHOR INFORMATION

### Corresponding Author

*Phone: +34927257122. Fax: +34927257110. E-mail: mariovet@unex.es.

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### Notes

The authors declare no competing financial interest.

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